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CHARACTERIZATION OF CELL LINES DEVELOPED FROM THE GLASSY-WINGED SHARPSHOOTER, *HOMALODISCA COAGULATA* (HEMIPTERA: CICADELLIDAE)

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Summary

Four continuous cell lines were established from the embryos of the glassy-winged sharpshooter, *Homalodisca coagulata* (Say), an economically important insect vector of bacterial pathogens of grape, almond, citrus, oleander, and other agricultural and ornamental plantings. The cell lines were designated GWSS-Z10, GWSS-Z15, GWSS-G3, and GWSS-LH. The GWSS-Z10, GWSS-Z15, and GWSS-G3 lines were cultured in Ex-Cell 401 medium supplemented with 10% fetal bovine serum (FBS), whereas the GWSS-LH line was cultured in LH medium supplemented with 20% FBS. The cell lines were characterized in terms of their morphology, growth, protein composition, and polymerase chain reaction–amplification patterns of their chromosomal deoxyribonucleic acid. The population doubling times of GWSS-Z10, GWSS-Z15, GWSS-G3, and GWSS-LH were 46.2, 90.9, 100.3, and 60.2 h, respectively. These lines should be useful for the study of insect-pathogenic viruses of leafhoppers, aphids, treehoppers, and other related insects as well as plant-pathogenic viruses that are transmitted by these insects.

Key words: GWSS; Pierce disease; *Xylella fastidiosa*; insect.

Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae), is an insect that feeds on the xylem fluid of plants. The GWSS appears to be native to the southeastern United States and northeastern Mexico (Triapitsyn and Phillips, 2000); however, it established itself as an invasive species in southern California during the 1990s (Blua and Morgan, 2003; Hoddle, 2004; Redak et al., 2004). The GWSS is an important agricultural pest insect because it can transmit bacterial pathogens such as *Xylella fastidiosa* during feeding (Purcell and Feil, 2001; Redak et al., 2004). *Xylella fastidiosa* induces disease in a number of high-value agricultural plantings including grape, almond, citrus, and stone fruit as well as ornamental plantings such as oleander, and forest trees such as oak, sycamore, elm, and maple. In particular, *X. fastidiosa* is the causative agent of Pierce disease (PD) of grapevines (Purcell and Feil, 2001; Redak et al., 2004). There is no known cure for PD or other *X. fastidiosa*–induced diseases. Pierce disease has been documented in California since the 1880s; however, during the 1990s, a new outbreak of PD was identified, which showed higher rates of spread (Purcell and Feil, 2001; Redak et al., 2004). These outbreaks were soon linked to the invasive GWSS, which in comparison with native Californian sharpshooters travels over greater distances at faster rates and has unique feeding behavior that is conducive for faster disease transmission.

In comparison with other sharpshooter species (a type of leafhopper), an abundance of data are available regarding the life cycle, behavior, nutritional requirements, habitat, pathogen transmission characteristics, etc. of GWSS. Much of these data have been collected in the field. A primary driver for the study of GWSS biology comes from the potential of GWSS-transmitted diseases to induce severe economic damage. This potential is particularly high in California because this state is by far the top producer of agricultural products in the United States with a total value of $27.8 billion in 2003 (Tolomeo and Rutz, 2004). Of this total, approximately 16% originates from grapes ($2.3 billion), almonds ($1.6 billion), and oranges ($0.5 billion), which are key hosts of GWSS. When value-added products such as wine or related industries such as tourism are considered, the potential economic effects of GWSS-vectored diseases are significantly magnified.

Although GWSS can be reared with some success in a greenhouse on single host plant species such as soybean, mass rearing of GWSS is difficult, and an artificial diet is not available for GWSS or other leafhopper species (Brodbeck et al., 2004). In addition, in most regions of the United States, appropriate containment facilities and governmental permits are required to rear GWSS. Thus, there are limitations in the use of GWSS as an in vivo model system. In this report, we describe the establishment and characterization of four cell lines derived from GWSS embryos that can be cultured in media that are commercially available or can be simply prepared in the laboratory.

Materials and Methods

Primary culture. Egg masses for the generation of primary cultures were collected from citrus groves near Filmore, Ventura County, California, during late April of 2001. The egg masses were stored at 5° C for up to 10 d before the generation of primary cultures. The primary cultures were generated from

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egg masses containing 11–16 eggs with embryos at a blastokinetic movement stage of development (*see* Hirumi and Maramorosch, 1964, 1971) in which eye discs were weakly visible. The egg masses were surface sterilized by immersion in 70% ethanol for 60 s, followed by three brief washes in sterile culture medium. After the sterilization and washes, the eggs were separated from the citrus leaf and placed into a 35-mm-diameter tissue culture dish (Falcon, Franklin Lakes, NJ) containing 2.0 ml of complete medium supplemented with antibiotics (kanamycin monosulfate [120 μg/ml] and penicillin [20 U/ml]–streptomycin [20 μg/ml][all from Sigma, St. Louis, MO]). The embryonic tissues were dissociated using sterile forceps, and 100 μl of this cell suspension was dispensed into the wells of a 96-well tissue culture plate (Costar #3596, Corning, NY, Falcon #353072, or Nunc #167008, Rochester, NY). Three media were tested for the generation of the primary cultures: Ex-Cell 401 (JRH Biosciences, Lenexa, KS), TNM-FH (Sigma) or LH (Basic Medium no. 1 for Leafhopper Cells [Hirumi and Maramorosch, 1971]) supplemented with 20% non–heat-inactivated fetal bovine serum (FBS, Gemini Bio-Products, Calabasas, CA) and antibiotics as described above. The primary cultures were maintained at 27° C.

Cell subculture. Once established, the GWSS cell lines were maintained in Ex-Cell 401 supplemented with 10% FBS or in LH supplemented with 20% FBS. Antibiotics were not added to these media. For subculture, the cells were detached from the 25-cm^2 culture flask (Falcon) by moderate spanking and pipetting. The detached cells were then mixed with fresh medium at a ratio of 1:1 to 1:5 into a new culture flask. For all the cell lines, trypsinization was performed infrequently but as necessary to detach the cells from the culture surface and to reduce clumping. In brief, the cells were washed twice with 5.0 ml of phosphate-buffered saline (PBS), pH 7.2 (GIBCO, Grand Island, NY), then 0.5 ml of 0.05% trypsin–ethylenediaminetetraacetic acid (EDTA) (GIBCO) was added to the flask. After a 5-min incubation at room temperature, 10–15 ml of fresh medium was added to the flask, and a uniform suspension of the detached cells was generated by gently pipetting. A 5.0-ml aliquot of this suspension was used to seed a new culture flask.

Light microscopy. Cell numbers and morphology were characterized by light microscopy using an inverted microscope (Olympus IMT2) and captured using a digital camera (Optronics MicroFire).

Measurement of cell growth. Cell growth was quantified using the trypan blue dye–exclusion hemacytometer method. In brief, 2.0 ml of a suspension of cells in fresh culture medium (approximately 5×10^5 cells/ml) was seeded into the wells of a six-well tissue culture plate (Falcon) or 35-mm-diameter culture dish (Falcon). At 24-h intervals, the medium from one well or dish was removed, the cells were washed twice with 2.0 ml of PBS buffer, pH 7.2, and 0.5 ml of 0.05% trypsin–EDTA was added. After a 5-min incubation at room temperature, the trypsin was inactivated by the addition of 0.5 ml of complete medium. Half a milliliter of this cell suspension was diluted with 0.5 ml of 0.4% trypan blue (Sigma), and the cell number was quantified using a hemacytometer. Population doubling times were calculated as described previously (Hayflick, 1973), where the doubling time in hours is equal to $72/(3.32\log N_2/N_1)$, where N_1 and N_2 are the cell numbers at 24 and 96 h after seeding, respectively.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting. Proteins from 1×10^4 cells or 300 μg of homogenized GWSS eggs were dissolved in Tris–glycine sodium dodecyl sulfate (SDS) sample buffer (Invitrogen, Carlsbad, CA) and separated by SDS–polyacrylamide gel electrophoresis (PAGE) using a precast 12% Tris–glycine gel (Invitrogen) as described by Laemmli (1970). After electrophoresis, the proteins were observed using GelCode Blue stain reagent (Pierce, Rockford, IL) or subjected to Western transfer (Burnette, 1981) to a 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA). After the electrophoretic transfer, the membrane was blocked using 5% nonfat dry milk in PBS–0.1% Tween 20 (PBS-T) for 1 h, washed with PBS-T (3×10 min), and hybridized with a 1:2000 dilution of the primary antibody (mouse anti-GWSS egg proteins, 1D4–1D8 [Hagler et al., 2002]) in PBS-T for 2 h. After the hybridization with the first antibody, the membrane was washed with PBS-T (3×10 min) and hybridized with a 1:5000 dilution of the secondary antibody (goat anti-mouse immunoglobulin conjugated to peroxidase, Sigma) in PBS-T for 1.5 h. After the second hybridization and final washes with PBS-T (3×10 min), the hybridized proteins were observed using an ECL Plus Western Blotting Detection Kit (Amersham, Piscataway, NJ) and BioMax MR film (Kodak, Rochester, NY). All the hybridizations and washes were performed at room temperature.

Genomic deoxyribonucleic acid analysis by polymerase chain reaction. The genomic deoxyribonucleic acids (DNAs) from each of the GWSS cell lines were isolated from 4×10^6 cells using a DNeasy Tissue Kit (Qiagen, Valencia, CA) and analyzed by a polymerase chain reaction (PCR). Genomic DNAs were also isolated from homogenized adult GWSS tissues (50 mg) or control cells (4×10^6) from the established lepidopteran cell lines Sf-21 from *Spodoptera frugiperda* (Vaughn et al., 1977) and High Five from *Trichoplusia ni* (Granados et al., 1994). The PCR was performed using Platinum *Taq* DNA polymerase (Invitrogen) and primers (Integrated DNA Technologies, Coralville, IA) Bm10S (5′-ATGGCCACCACAAATGC-3′), Bm12S (5′-ATGTACAGGCACGCGTG-3′), Bm19S (5′-ATGAATAGCGGCGACG-3′), Bm81A (5′-CGCCGCTATCGATGA-3′), and Bm92A (5′-TGATACCGCTGACGCC-3′) in the combinations described in the legend for Fig. 4 under the conditions recommended by the manufacturer. The primers Bm10S, Bm12S, Bm19S, Bm81A, and Bm92A were originally designed to recognize open reading frames 10, 12, 19, 81, and 92, respectively, of the genome of the baculovirus *Bombyx mori* nucleopolyhedrovirus (BmNPV) (Gomi et al., 1999). In general, PCR amplification was performed as follows: 95° C for 3 min; 30 cycles at 95° C for 45 s, 45° C for 1 min, and 72° C for 1 min; and 72° C for 10 min. The PCR-amplified products were separated by 0.7% agarose gel electrophoresis and observed by staining with ethidium bromide. Molecular weight markers, 1-kbp DNA ladder, were from Promega (Madison, WI).

Results

Primary cultures. The primary cultures were generated from GWSS egg masses in May 2001. Three different media (Ex-Cell 401, TNM-FH, and LH, each supplemented with 20% FBS and antibiotics) and three types of tissue culture–treated 96-well plates (from Costar, Falcon, and Nunc) were used to start the primary cultures. During the initial 24–48 h, a large amount of cell lysis was observed under all the conditions tested. Foci of spindle-shaped and round cells as well as sheets of epithelial-like cells that were loosely attached to the culture surface were observed during the next 2 wk. After the initial 2 wk, an equal volume of medium was added to each of the wells. Subsequently, as the cell number increased, the culture plate was carefully tilted to one side and 100% of the medium was replaced at 10–14 d intervals for an additional 4 to 6 wk. At this point, the cells in wells that showed the strongest growth were transferred to the wells of a 12-well plate (Falcon) and subsequently to the wells of a six-well plate (Falcon) or culture tube (Nunc). This process took an additional 3–4 mo. This process preferentially selected for attached cells because the cells in suspension were lost during passage. The medium was replaced at 10–14 d intervals during this period. The Ex-Cell 401 and LH media gave the best results for starting and maintaining the primary cultures. There was no apparent difference between the tissue culture–treated plasticware from the three manufacturers. Primary cultures that were started from egg masses in which the embryos were at very early (i.e., eye discs were not clearly visible) or very late (i.e., eye discs were clearly visible at the anterior of the embryo) developmental stages did not generate viable primary cultures. In addition, primary cultures that were maintained at the higher temperature of 30° C died within 2 wk.

Cell lines. About 6 mo after the start of the primary cultures, the cells were adapted to Ex-Cell 401 medium containing lower concentrations of FBS and no antibiotics. Two approaches were concurrently taken. First, the FBS concentration was lowered from 20 to 10% in a single step, and second, the FBS concentration was lowered from 20 to 15 to 10% during a 2-mo period. The first approach generated a cell line, GWSS-Z10, which was composed of spindle-shaped and spherical cells (Fig. 1*A*). The second approach generated a cell line, GWSS-Z15, which was composed primarily of spindle-shaped cells (Fig. 1*B*). The same primary culture was used to generate the GWSS-Z10 and GWSS-Z15 lines.

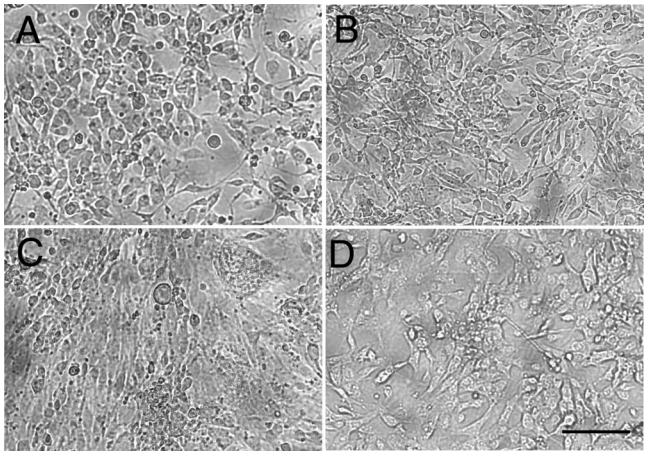

FIG. 1. Photos showing the morphology of the cell lines established from embryonic tissues of the glassy-winged sharpshooter. The images are from GWSS-Z10 (*A*), GWSS-Z15 (*B*), GWSS-LH (*C*), and GWSS-G3 (*D*). The approximate passage numbers of the GWSS-Z10, GWSS-Z15, GWSS-LH, and GWSS-G3 lines were 105, 71, 80, and 50, respectively. All the images are at the same magnification. The *bar* in *panel D* indicates approximately 100 μm.

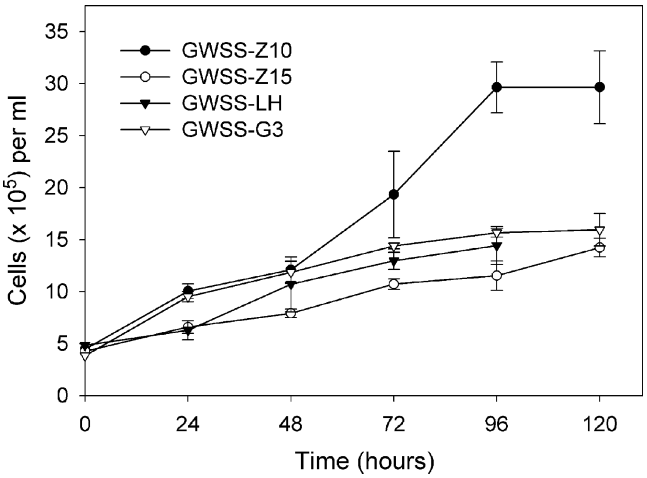

FIG. 2. Growth curves of the glassy-winged sharpshooter cell lines GWSS-Z10, GWSS-Z15, GWSS-LH, and GWSS-G3. The error *bars* indicate the standard deviation of three or four independent trials. The approximate passage numbers of the GWSS-Z10, GWSS-Z15, GWSS-LH, and GWSS-G3 lines were 148, 100, 117, and 87, respectively.

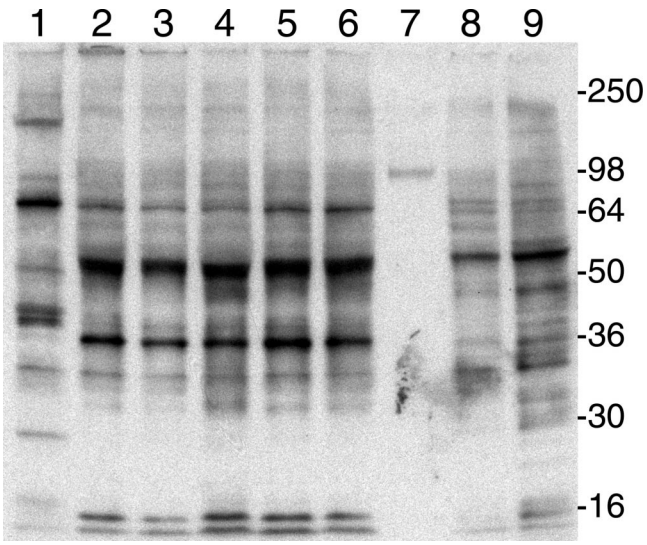

FIG. 3. Western blot analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12%) separated proteins from homogenized glassy-winged sharpshooter (GWSS) eggs (1), GWSS-Z10 (2), GWSS-Z15 (3), GWSS-LH (4), GWSS-R10 (5), GWSS-G3 (6), SeeBlue marker (7), Sf-21 (8), and High Five (9). The molecular masses in kiloDaltons of the SeeBlue markers are indicated to the right. The proteins were hybridized with antibodies generated in a mouse against GWSS egg proteins. *Note:* The GWSS-R10 cell line has since been lost during passage. The approximate passage numbers of the GWSS-Z10, GWSS-Z15, GWSS-LH, and GWSS-G3 lines were 61, 41, 50, and 21, respectively.

The GWSS-LH cell line was composed mostly of spindle-shaped and spherical cells that grow in tightly packed monolayers that strongly attach to culture surface (Fig. 1*C*). These cells are growing in LH medium supplemented with 20% FBS without antibiotics. An attempt was also made to adapt these cells to LH medium containing 10% FBS in a single step; however, this attempt was unsuccessful.

To generate "subclones" of the GWSS-Z10 line, GWSS-Z10 cells were diluted in Ex-Cell 401 containing 10% FBS and dispensed into the wells of a 96-well tissue culture plate (Costar). Each well contained approximately 20 cells in 200 μl of medium. After 4 wk, the cells in four of the wells showed moderate to strong growth and the medium was replaced in these wells. One week later, the cells in each of these wells was diluted in fresh medium and dispensed in the wells of new 96-well tissue culture plates (Nunc). Each well contained less than 20 cells in 200 μl of medium. After another 4 wk, medium to strong cell growth was found in five of the wells (B3, E5, E9, G3, and G4), and the medium in these wells was replaced. A week later, the cells in these wells were transferred individually to the wells of a 48-well tissue culture plate (Falcon). After two additional months of medium changes and subcultures in the 48-well culture plates, only cells that originated from well G3 continued to proliferate. This line was designated GWSS-G3. The GWSS-G3 cell line was composed mostly of spindle-shaped cells that strongly attached to the culture surface (Fig. 1*D*).

Cell growth. Growth curves that are typical of those generated by the four GWSS cell lines are shown in Fig. 2. The GWSS-Z10 line showed significantly faster growth in comparison with the other lines. The population doubling times of GWSS-Z10, GWSS-Z15, GWSS-LH, and GWSS-G3 were 46.2 ± 1.6, 90.9 ± 14.0, 60.2 ± 4.9, and 100.3 ± 6.8 h, respectively.

Western blot analysis. The GWSS-specific protein composition of each of the GWSS cell lines was analyzed by Western blot analysis (Fig. 3) using an antibody that was generated against GWSS egg proteins. The antibody recognized two major (ca. 35 and 50 kDa) and numerous minor proteins from each of the GWSS cell lines. The sizes and intensities of the proteins that were recognized from each of the four GWSS lines were highly similar. In contrast, the antibody recognized three major (ca. 38, 40, and 65 kDa) and numerous minor proteins from the homogenized GWSS eggs. In general, the proteins that were recognized from the GWSS cell lines were different (in terms of both size and expression level) from those recognized from the lepidopteran-derived Sf-21 and High Five cell lines. The proteins that were recognized from the GWSS cell lines

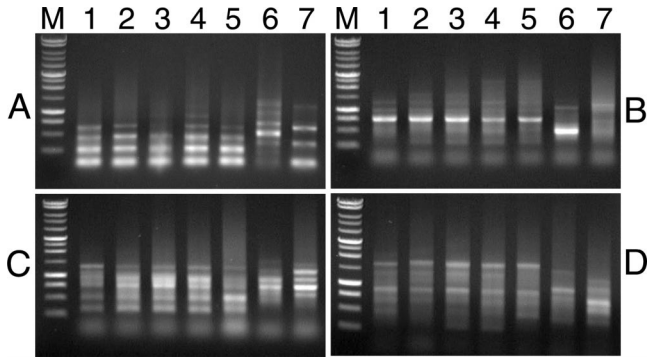

FIG. 4. Polymerase chain reaction amplification products of genomic deoxyribonucleic acid (DNA) templates amplified with primers Bm10S and Bm12S (*A*), Bm12S and Bm19S (*B*), Bm81A and Bm12S (*C*), and Bm92A and Bm12S (*D*). The genomic DNAs were isolated from homogenized adult glassy-winged sharpshooter (*lanes 1*) or cells of GWSS-Z10 (*lanes 2*), GWSS-Z15 (*lanes 3*), GWSS-LH (*lanes 4*), GWSS-G3 (*lanes 5*), Sf-21 (*lanes 6*) or High Five (*lanes 7*). *Lanes M*: 1-kbp DNA ladder (10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, 1.0, 0.75, 0.5, and 0.25 kbp). The approximate passage numbers of the GWSS-Z10, GWSS-Z15, GWSS-LH, and GWSS-G3 lines were 134, 90, 94, and 65, respectively.

and homogenized eggs were more similar in terms of size; however, their expression levels appeared to be different. A similar pattern was found when the total protein composition of the GWSS cell lines, GWSS eggs, and control lepidopteran cell lines were compared by GelCode Blue staining of SDS-PAGE separated proteins (data not shown).

Genomic DNA analysis. Genomic DNAs isolated from each of the four GWSS cell lines as well as adult GWSS were analyzed by PCR using four different primer pairs (Fig. 4). The patterns of the PCR-amplified DNA fragments were highly similar among the four GWSS cell lines and adult GWSS but different from those generated from Sf-21 and High Five genomic DNAs.

DISCUSSION

In this report, we characterize four insect cell lines that we established from GWSS embryos collected in the field. Western blot analysis indicated that the protein composition of each of these cells lines was different from that found in GWSS eggs from laboratory-reared adults. Thus, we initially thought that our field-collected egg masses might have originated from another sharpshooter such as the native Californian blue-green or smoke tree sharpshooter rather than from GWSS. However, this was not the case because comparison of the genomic DNAs from adult GWSS and from our cell lines indicated that their genotypes were essentially identical. In addition, closer inspection of the Western blot and protein staining results indicated that differences in protein expression levels were also factors in the differences in the protein patterns.

The use of different media and adaptation strategies to lower the serum concentrations in the media resulted in lines that were different in terms of morphology and growth characteristics. Lowering the serum concentration quickly, rather than incrementally during a 2-mo-long period generated a line, GWSS-Z10, with the fastest doubling time.

Insect baculoviruses such as *Autographa californica* nucleopolyhedrovirus (AcMNPV) are known to enter (although not produce progeny) a wide variety of cells from insect-derived (Miller and Lu, 1997) and non–insect-derived (Hofmann et al., 1995; Leisy et al., 2003) cell lines. To test whether baculoviruses could enter and possibly replicate in cells of GWSS origin, GWSS-Z10 cells were inoculated with wild type AcMNPV or a recombinant AcMNPV carrying a *lac*Z marker cassette (β-galactosidase gene driven by a heat shock promoter [*hsp70*] from *Drosophila melanogaster*) at a multiplicity of infection of 10 plaque-forming units per cell. Baculovirus-inoculated GWSS-Z10 cells did not display any virus-induced cytopathic effects (CPE) for up to 5 d after inoculation; however, β-galactosidase was detected in the recombinant AcMNPV-inoculated cells (data not shown) indicating the baculovirus is able to enter hemipteran cells. Similar results were found when a different baculovirus strain (BmNPV) carrying the *lac*Z marker cassette was tested (data not shown). Although baculoviruses were unable to replicate in the GWSS cells, *Rhopalosiphum padi* virus, an ribonucleic acid virus isolated from the bird cherry-oat aphid (Hemiptera: Aphididae) induces CPE and appears to replicate in GWSS-Z10 and GWSS-Z15 cells (S. Boyapalle, W. A. Miller, and B. C. Bonning, pers. comm.).

Of the more than 450 insect cell lines that have been established (Lynn, 1999), only a handful is of leafhopper origin (e.g., Hirumi and Maramorosch, 1971; Hink, 1976; Omura and Kimura, 1994; Wayadande and Fletcher, 1998). To the best of our knowledge, the lines described here are the first and only ones to be established from GWSS. These lines should be of use not only for the study of the basic biology of GWSS but also for the study of insect-pathogenic viruses of leafhoppers, aphids, treehoppers, and other related insects as well as plant-pathogenic viruses that are transmitted by these insects.

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